The Missing C-17 O-Methyltransferase in Geldanamycin Biosynthesis

Min Yin,[†,§] Tao Lu,[†] Li-Xing Zhao,[†] Yihua Chen,[⊥] Sheng-Xiong Huang,[§] Jeremy R. Lohman,[§] Li-Hua Xu,[†] Cheng-Lin Jiang,[†] and Ben Shen*[§,∥,‡,⊥]

[†]Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan 650091, China; [§]Department of Chemistry, [∥]Department of Molecular Therapeutics, and [‡]Natural Products Library Initiative at TSRI, Scripps Florida, Jupiter, FL 33458, United States; and [⊥]Division of Pharmaceutical Sciences, School of Pharmacy, University of Wisconsin-Madison, Madison, WI 53705, United States.

shenb@scripps.edu

Supporting Information

General experimental procedures

Bacterial strains, plasmids, and media. *Escherichia coli* DH5α[1] was used for routine cloning, *E. coli* XL1 Blue MR and Gigapack III XL (Stratagene, La Jolla, CA) for genomic library construction, *E. coli* BW25113/pIJ790 for PCR-targeting and λ-RED-mediated mutagenesis,[2] and *E. coli* ET12567/pUZ8002[3] for *E. coli-S. autolyticus* conjugation. *S. autolyticus* CGMCC 0516 has been described previously.[4] The pGEM-T easy vector (Promega, Madison, WI), pET-28a(+) (Novagen, Madison, WI), pMD18T (Takara, Shiga, Japan), and pSmart (Lucigen, Madison, WI) were from commercial sources. pIJ773,[2] pBS9010,[5] and pUWL201PW[6] were described previously. pHAQ34 is a modified version of SuperCos 1 (Stratagene), in which the neomycin resistance marker has been replaced with the thiostrepton resistance gene and the oriT fragment. pHY773 is a modified version of pIJ773, in which the oriT fragment has been deleted. Ampicillin (100 mg mL^{-1}), apramycin (50 mg mL^{-1}), and chloramphenicol (25 mg mL^{-1}) were used for selection in *E. coli*. Apramycin (50 mg mL^{-1}) and thiostrepton (10 mg mL^{-1}) were used for selection of *S. autolyticus* recombinants. Nalidixic acid (50 mg mL^{-1}) was used to select against the *E. coli* conjugal donors after *E. coli-S. autolyticus* conjugation.

S. autolyticus CGMCC wide-type, the *ΔgdmAI* mutant strain SB17001, the *ΔgdmMT* mutant strain SB17002, and the *ΔgdmMT* mutant complemented strain SB17003 were routinely grown at 28°C in TSB liquid medium. ISP4 solid medium was used to prepare spores, and IWL4 solid medium (ISP4 medium supplemented with 0.05% yeast extract, 0.1% tryptone, and $MgCl_2$ to a final concentration of 20 mM)[7] was used to plate out conjugation mixtures. For GDM and analogs production, fresh spores of *S. autolyticus* and recombinant strains were cultured in 250-mL baffled flasks containing 50 mL of TSB medium for 40 hrs at 28°C and 250 rpm. Then, 0.5 mL of the seed cultures were inoculated into 250-mL baffled flasks containing 50 mL of the production medium (2% soya flour, 0.2%peptone, 2% glucose, 0.5% soluble starch, 0.2% yeast extracts, 0.4% NaCl, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.2% $CaCO_3$) and fermented at 28°C, 250 rpm for 5 days.

General instrumentation. Unless otherwise noted, PCR experiments were performed with LA Taq polymerase (Takara Bio Inc, Shiga, Japan) and run in an Eppendorf Gradient Thermocycler (Brinkman Instruments, Westbury, NY). High performance liquid chromatography (HPLC) was performed with an Apollo C18 column (5 µm, 4.6 x 250 mm; Grace Davison Discovery Sciences, Deerfield, IL) (analytical) or an Alltima C18 column (5 µm, 10 x 250 mm; Grace Davison Discovery Sciences) (semi-prep) on a Varian Liquid Chromatography System (Varian, Walnut Creek, CA) consisting of Varian ProStar 210 pumps and a ProStar 330 photodiode array detector. Electrospray ionization-mass (ESI-MS) spectra were acquired on an IonSpec HiResMALDI FT-Mass spectrometer with a 7 tesla superconducting magnet. ^1H and ^{13}C NMR spectra were recorded at 25°C on a Varian Unity Inova 500 instrument operating at 500 MHz for ^1H and 125 MHz for ^{13}C nuclei.

DNA isolation, manipulation, and *E. coli-S. autolyticus* conjugation. DNA isolations and manipulations in *E. coli* and *Streptomyces* were carried out according to standard procedures.[1,3] For Southern analysis, digoxigenin labeling of DNA probes, hybridization, and detection were performed according to the protocols provided by the manufacturer (Roche Diagnostics Corp.,

Indianapolis, IN, USA). *E. coli–S. autolyticus* conjugation was carried out using standard literature conditions with the following minor modifications: (i) *S. autolyticus* spores in 2 x YT medium were first incubated at 50°C for 10 min followed by 5 hrs at 30°C before mixing with the *E. coli* ET12567/pUZ8002 donors and (ii) the resultant mixed culture was grown on IWL4 medium. The plates were incubated for 18 hrs at 30°C before they were overlaid with 1 mL H_2O containing final concentrations of 25 μg/mL nalidixic acid (to select against *E. coli*) and 50 μg/mL apramycin (to select for exconjugants). The plates were incubated for 8-10 days at 30°C. The resultant colonies were replica-plated to isolate the apramycin-resistant and thiostrepton-sensitive clones that had undergone double crossover homologous recombination to replace the targeted gene.

Cloning, sequencing, and annotation of the GDM biosynthetic gene cluster. A cosmid library of *S. autolyticus* chromosomal DNA was constructed by partial digestion with *Sau*3AI, dephosphorylation and ligation into the *Bam*HI site of pHAQ34. The ligation mixture was packaged with Gigapack III XL packaging extract and transduced into *E. coli* XL1 Blue MR cells. Using primers designed according to known *gdmN* genes,[8] a fragment of *gdmN* was amplified by PCR from *S. autolyticus* using the following pair of primers (forward primer 5'-AAGGTGATGGGCCTGGCGCC-3' and reverse primer 5'-CGCGCGGTGCCGTCCACATG-3'), cloned into pMD18-T to afford pBS17004, and confirmed its identity as *gdmN* gene by DNA sequencing. A 0.88-kb *Pst*I-*Eco*RI fragment of *gdmN* was then cloned from pBS17004 and digoxigenin labeled as probe 1. The genomic library was screened by colony hybridization with probe 1, and positive clones were confirmed by end sequencing. A 0.53-kb *gdmAI* fragment was then PCR-amplified from pBS17002 using the following pair of primers (forward primer 5'-TGCTGAGGCTGGATTGGG-3' and reverse primer 5'-ACAGAACCAGGATCAGGAGACC-3'), cloned into pMD18-T to afford pBS17005 and digoxigenin labeled as probe 2 for chromosomal walking to isolate overlapping cosmids covering the entire cluster. Three overlap cosmids (pBS17001, pBS17002, pBS17003) were chosen for complete sequencing. Subclones library was constructed by inserting 1-4 kb cosmids fragments into pSmart and sequenced by ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). SeqScape (Applied Biosystems, Foster City, CA) was used for sequence assembling. ORF assignments, sequence alignments, and identity and similarity calculations were made with the assistance of ORF finder, glimmer, and BLAST from NCBI websites, respectively.

Inactivation of *ΔgdmAI* to isolate the *ΔgdmAI* mutant strain SB17001. The 1.1-kb gene disruption *aac*(3)*IV* cassette ($Apra^R$ gene) was PCR amplified from pHY773 using the following pair of primers (forward primer 5'-ATGCTACGGACTGACCTGATCCGGCCGGTGCCCGAACTGATTCCGGGGATCCGTCGACC-3' and reverse primer 5'-CCGCAGCCGTGCG GGCAGCTCAAGCAGCACATGCCGGGTTGTAGGCTGGAGCTGCTTC-3'), and the resultant fragment was then introduced, by electroporation, into *E. coli* BW25113/pIJ790 that harbored the *gdmAI* containing cosmid pBS17002. The mutated *ΔgdmAI* cosmids were isolated and introduced into *S. autolyticus* by intergeneric conjugation. $Apra^R$ $Thio^S$ exconjugants were picked out as double crossover mutants, and screened by comparing the size of PCR products using the following pair of primers (forward primer 5'-CTCGCACAGGCCGGATAT-3' and reverse primer 5'-CTCCACGAGAACGGCAGATA-3'). Subsequent southern blot confirmed successful

construction of the *ΔgdmAI* mutant strain SB17001 (Figure S2). The primers used to prepare the probe for Southern analysis were: forward primer 5'-GTCGGGTGTCCTCGCCTTGA-3' and reverse primer 5'-GGGAGTTTGGCCCGTTTGGT-3'.

Inactivation of *ΔgdmMT* to isolate the *ΔgdmMT* mutant strain SB17002. The *ΔgdmMT* mutant strain SB17002 was constructed in a similar way as SB17001 with the following primers used to amplify the *ΔgdmMT* gene disruption cassette: forward primer 5'-CTATCCGGTGACCGTGTCGACCCTGAAGGAGATCGCATGATTCCGGGGATCCGTCGACC-3' and the reverse primer 5'-GGCCGCCCCCGG GAACAGCGCCGTCCAGCGGAGCGCTCATGTAGGCTGGAGCTGCTTC-3'. The desired *ΔgdmMT* mutant strain SB17002 was isolated by PCR screening and confirmed by Southern hybridization (Figure S3). The primers used for PCR screening for the desired mutants were: forward primer 5'-TGCACCCCGACTACCAGG-3' and reverse primer 5'-CGCCGGAAGAAGAATCTATCC-3'. The primers used to prepare the probe for Southern analysis were: forward primer 5'-GCGACTGGTCGAAGTGGTGTTC-3' and reverse primer 5'-CGGTGGAGCGGCAGATTGA-3'.

The *ΔgdmMT* mutant complementation strain SB17003. To complement the *ΔgdmMT* mutation in SB17002, the *gdmMT* gene fragment was PCR amplified from pBS17003 with the following pair of primers (forward primer 5'-GGAATTCCATATGGGGCCTGAGATCGAGATTGC-3' and reverse primer 5'-CGGGATCCTCAGCCGAGCCGCACACCCGCCAGGATG-3'), which introduced the *Eco*RI/*Nde*I and *Bam*HI sites at its N- and C-termini, respectively. After *Eco*RI and *Bam*HI digestion, the resultant fragment was inserted into the same sites of pBS9010 to afford pBS17006. The *gdmMT* gene was then cloned as an *Nde*I-*Bam*HI fragment from pBS17006 and inserted into the same sites of pUWL201PW to afford pBS17007. Finally, the *gdmMT* gene was moved together with the *ErmE** promoter from pBS17007 as an *Sma*I-*Bam*HI fragment and inserted into the same sites of pBS9010 to yield pBS17008, in which the expression of *gdmMT* is under the control of *ErmE**.[3] Introduction of pBS17008 into SB17002 by conjugation finally afford the complementation strain SB17003.

Isolation of 2 and 4. Fermentation cultures of SB17002 were centrifuged at 3500 x g for 30 min, the resulting supernatant was extracted with EtOAc twice. The extract was subjected to RP-18 silica gel chromatography with stepwise methanol-water gradient elution (0%, 25%, 50%, 75% and 100%). The 50% methanol elution was combined and evaporated to dryness. Semi-preparative HPLC was finally used to isolate pure **2** and **4** on a Varian liquid chromatograph system with an Alltima-C18 column (5 μm, 10 mm x 25 cm). The column was equilibrated with 70% solvent A (H_2O) and 30% solvent B (acetonitrile) and developed with a linear gradient (0-25 min, from 30% B to 100% B) and then kept 100% B for 5 min at a flow rate of 3 mL min^{-1} and UV detection at 304 nm using a Varian Prostar 330 PDA detector.

Overproduction in *E. coli* and purification of GdmMT to homogeneity. The *gdmMT* gene was PCR amplified from pBS17003 using the following pair of primers that introduced the *Nde*I and *Hind*III sites at its N- and C-termini, respectively (forward primer 5'-GGAATTCCATATGCCGTCCACACTGCACAC-3' and the reverse primer

5'-CCCAAGCTTCAGCCGAGCCGCACACCCG-3'). The resultant PCR product was inserted into pGEM-T, and the fidelity of *gdmMT* was confirmed by DNA sequencing. The *gdmMT* gene was then recovered as a *Nde*I-*Hind*III fragment and inserted into the same sites of pET-28a(+) to afford the expression construct pBS17009. To express *gdmMT* in *E. coli*, pBS17009 was transformed into *E. coli* BL21, and the resultant recombinant strains were cultured in LB medium, supplemented with 50 μg mL^{-1} of kanamycin, at 30°C, 250 rpm until A_{600} reached 0.6. At this time, 0.5 mM isopropyl 1-thio-β-D-galactopyranoside was added and fermentation continued under same conditions for additional 5-6 hrs. All the following purification operations were performed at 4°C. Cells were harvested by centrifugation at 3000 x g (Sorvall legent RT 75006441K rotor, Thermo Fisher Scientific Inc., Waltham, MA) and the cell pellets were re-suspended in lysis buffer (100 mM potassium phosphate, pH 7.0, 300 mM NaCl, 10 mM imidazole, 10% Glycerol) and lysed by sonication. After centrifuged at 12000 x g for 30 min, the resulting supernatant was loaded onto a pre-equilibrated HisTrapTM HP column (GE Healthcare). After washed by 10 volumes of wash buffer (50 mM phosphate, pH 7.0, 50 mM NaCl, 40 mM imidazole, 10% Glycerol), GdmMT was eluted with 3 volumes of elution buffer (50 mM phosphate, pH 7.0, 50 mM NaCl, 300 mM imidazole, 10% Glycerol). The resulted sample were concentrated with ultrafiltration centrifugation tubes (Ultracel series 10-kDa; Millipore) and desalted with PD-10 desalting columns (GE Healthcare). The purified proteins were examined on 12% SDS-PAGE.

Enzymatic assays of GdmMT as the C-17 O-methyltransferase. The C-17 O-methyltransferase activity of GdmMT was assayed at 30°C in 100 μL mixtures (50 mM potassium phosphate, pH 7.0, 50 mM NaCl, 5 mM $MgCl_2$, 400 μM SAM, 100 μM **4**). The reactions were started by adding GdmMT to a final concentration of 20 μM and terminated by adding 2 μL of trifluoroacetic acid. Identical assays with boiled GdmMT or without GdmMT were carried out as negative controls. The pH dependence of GdmMT was investigated in different buffers (50 mM potassium phosphate, pH 5.5-8.0 and 50 mM bis-tris-HCl, pH 8.0-9.5). Influences of different divalent metal ions (5 mM Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} with Cl^-) on GdmMT activity was investigated under the same conditions as described above in 50 mM potassium phosphate, pH 7.0.

HPLC analysis of 1, 2, and 4. Culture broths of *S. autolyticus* wild-type or recombinant strains were extracted with Amberlite XAD-16 resins and analyzed by a Varian liquid chromatograph system with an Apollo C18 column (5 μm, 250 × 4.6 mm). The column was equilibrated with 70% solvent A (H_2O) and 30% solvent B (acetonitrile) and developed with a linear gradient (0-25 min, from 30% B to 100% B) and then kept 100% B for 5 min at a flow rate of 1 mL min^{-1} and UV detection at 304 nm using a Varian Prostar 330 PDA detector.

17-O-Demethyl-GDM (2): Yellow solid; HR-ESI-MS (positive ion) for the $[M+Na]^+$ ion at *m/z* 569.24725 (calcd for $C_{28}H_{38}N_2O_9Na$, 569.24750); ^1H NMR and ^{13}C NMR spectra are consistent with those in the literature (see Figures S4 and S5).[9]

17-Amino-17-demethoxy-GDM (4): Yellow solid; HR-ESI-MS (positive ion) for the $[M+Na]^+$ ion at *m/z* 568.26342 (calcd for $C_{28}H_{39}N_3O_8Na$, 568.26348); ^1H NMR and ^{13}C NMR are consistent with those in the literature (see Figures S6 and S7).[10]

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Table S1. Deduced functions of ORFs of the GDM cluster[a] from *S. autolyticus* CGMCC 0516[b] in comparison with the ORFs of the three GDM clusters[a] cloned previously from *S. hygroscopicus* NRRL 3602,[c] *S. hygroscopicus* JCM 4427,[d] and *S. hygroscopicus* 17997,[e] respectively

CGMCC 0516[b]		NRRL 3602[c]		JCM 4427[d]		17997[e]			Homology (3602/4427/17997)	
Gene	#AA	Gene	#AA	Gene	#AA	Gene	#AA	Function	% identity	% similarity
orf1-orf13		*orf1-orf20*		*orf1-orf19*						
gdmMT	225							C-17-O-methyltransferase		
orf14-orf31		*gdmL*								
		gdmX								
gdmAI	6840	*gdmAI*	6841	*gelA*	6855			Type I PKS	89/89/-	92/92/-
gdmAII	3418	*gdmAII*	3434	*gelB*	3432			Type I PKS	88/88/-	91/91/-
gdmAIII	3872	*gdmAIII*	3895	*gelC*	3888	*gdmAIII*	3053	Type I PKS	89/89/100	89/89/100
gdmF	257	*gdmF*	257	*gelD*	257	*gdmF*	257	Amide synthase	95/95/100	96/96/100
gdmM	540	*gdmM*	544	*gel7*	538	*gdmM*	547	Flavin-dependent monooxygenase	89/88/99	93/93/100
orf32	98	*orf32*	98	*orf32*	98	*orf32*	98	Unknown	86/87/100	88/89/100
gdmN	685	*gdmN*	682	*gel8*	685	*gdmN*	683	Carbamoyltransferase	93/93/99	96/96/100
gdmH	370	*gdmH*	370	*gel9*	370	*gdmH*	370	Methoxymalonyl-ACP biosynthesis	92/92/100	94/94/100
gdmI	370	*gdmI*	370	*gel10*	370	*gdmI*	370	Methoxymalonyl-ACP biosynthesis	93/92/99	95/95/99
gdmJ	91	*gdmJ*	91	*gel11*	91	*gdmJ*	91	Methoxymalonyl-ACP biosynthesis	90/90/100	96/96/100
gdmK	288	*gdmK*	288	*gel12*	288	*gdmK*	288	Methoxymalonyl-ACP biosynthesis	94/93/100	94/94/100
gdmG	218	*gdmG*	218	*gel13*	218	*gdmG*	218	Methoxymalonyl-ACP biosynthesis	96/96/100	99/99/100
gdmRII	927	*gdmRII*	926	*gel14*	926	*gdmRII*	927	Transcriptional regulator	91/91/100	94/94/100
gdmO	354	*gdmO*	354	*gel15*	354	*gdmO*	354	Amino DHQ synthase	92/92/100	95/95/100
gdmFdx	64	*gdmFdx*	65	*gdmFdx*	65	*gdmFdx*	64	Ferredoxin	95/95/100	97/97/100
gdmP	400	*gdmP*	397	*gel16*	397	*gdmP*	400	P450 oxygenase	94/94/99	95/95/99
gdmRI	981	*gdmRI*	962	*gel17*	962	*gdmRI*	968	Transcriptional regulator	84/84/99	89/89/99
orf33-orf44		*orf22*								

[a]The core GDM biosynthetic gene cluster consists of 17 ORFs ranging from *gdmAI* to *gdmRI*, and these ORFs are conserved among the four cloned gene clusters (the GDM cluster from *S. hygroscopicus* 17997 was incomplete). Also see Figure S1.
[b]A total of 104,517 bp sequenced and deposited to GenBank under accession number HQ840740.
[c]A total of 85,375 bp was sequenced and reported,[8] but only 69,644 bp of the sequenced region was deposited to GenBank under accession number AY179507. *orf32* and *gdmFdx* were not annotated in the original submission and were done in the current work.
[d]A total of 75,920 bp sequenced and deposited to GenBank under accession number DQ249341. Only four genes, gelA, B, C, D, were annotated in the original submission, and the rest were annotated in the current work.
[e]A total of 28,356 bp sequenced and deposited to GenBank under accession number DQ914285. The GDM cluster was incomplete starting from partial sequence of *gdmAIII*, and *gdmAIII* is incomplete. *orf32* and *gdmFdx* were not annotated in the original submission and were done in the current work.

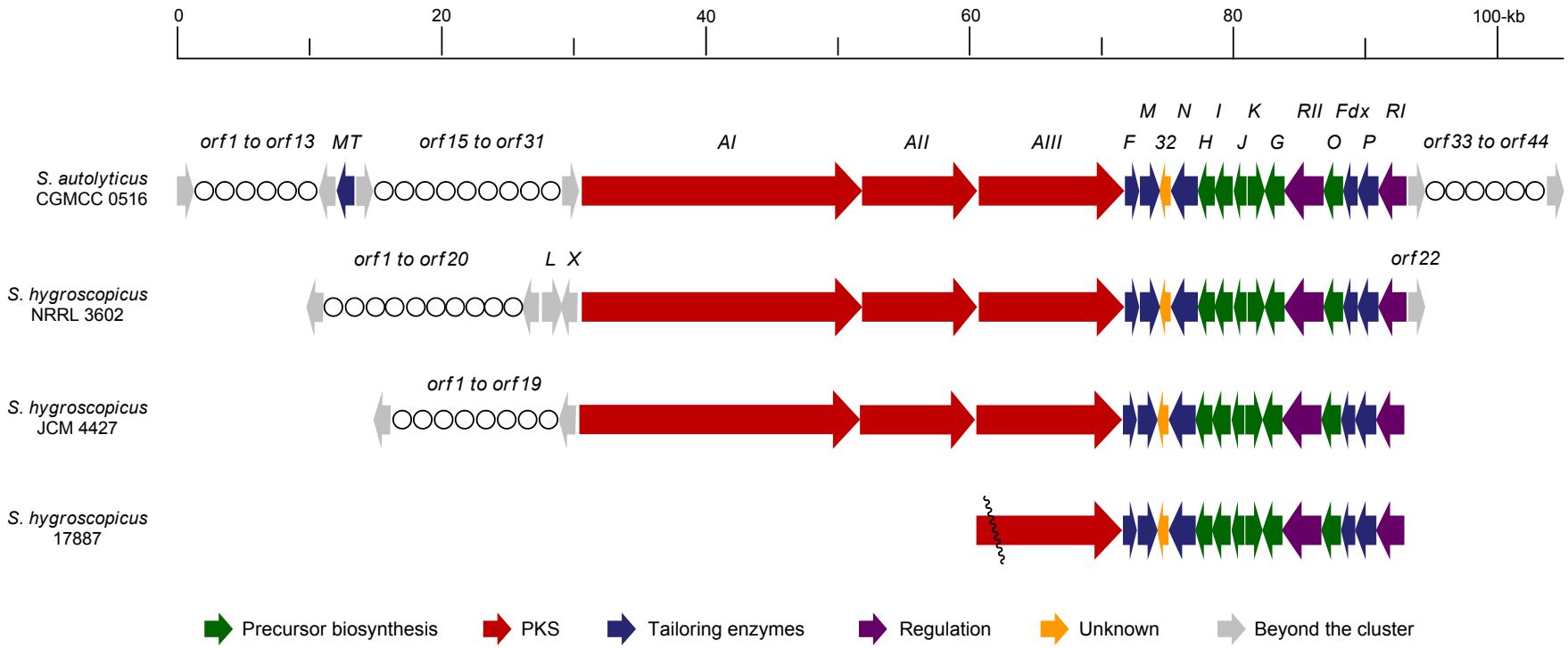
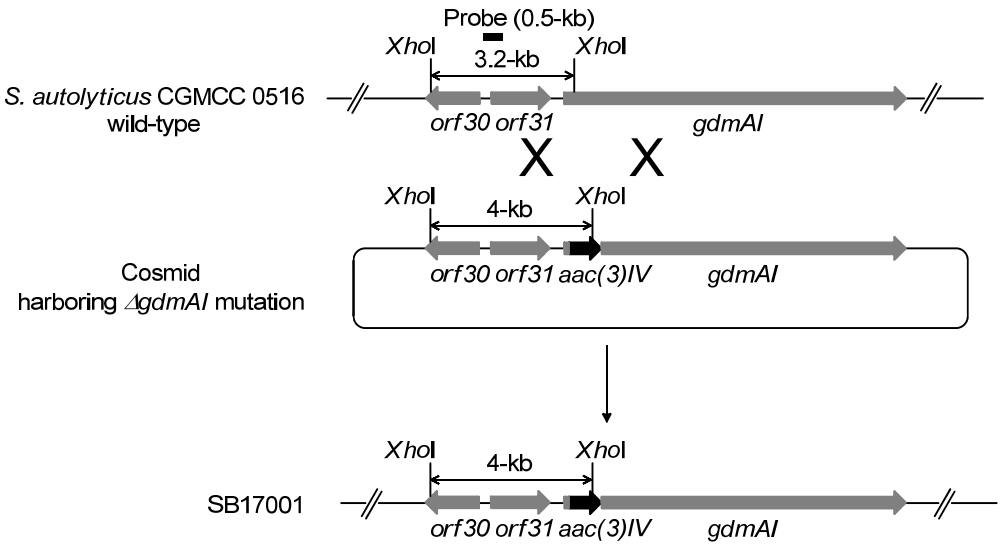


Figure S1. Genetic organization of the GDM cluster from *S. autolyticus* CGMCC 0516 and comparison to the three GDM clusters cloned previously from *S. hygroscopicus* NRRL 3602, *S. hygroscopicus* JCM 4427, and *S. hygroscopicus* 17997 (incomplete). See Table S1 for ORF functional assignments.

A



B

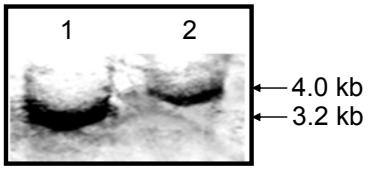
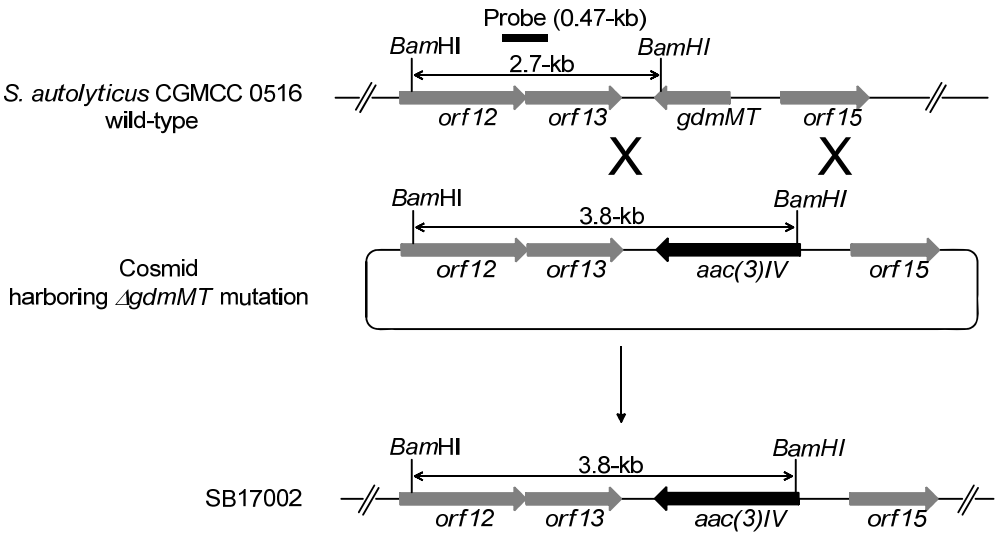


Figure S2. (A) Construction of the *⊿gdmAI* mutant strain SB17001 and restriction maps of *S. autolyticus* CGMCC 0516 wild-type and SB17001 strains showing fragment sizes upon *Xho*I digestion. (B) Southern analysis of *S. autolyticus* CGMCC 0516 wild-type (lane 1) and SB17001 (lane 2) genomic DNA digested with *Xho*I using the 0.5-kb PCR-amplified fragment as a probe.

A



B

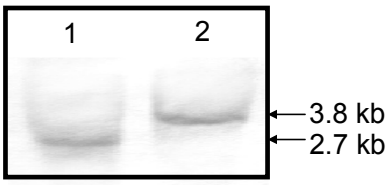


Figure S3. (A) Construction of the *ΔgdmMT* mutant strain SB17002 and restriction maps of *S. autolyticus* CGMCC 0516 wild-type and SB17002 strains showing fragment sizes upon *Bam*HI digestion. (B) Southern analysis of *S. autolyticus* CGMCC 0516 wild-type (lane 1) and SB17002 (lane 2) genomic DNA digested with *Bam*HI using the 0.47-kb PCR-amplified fragment as a probe.

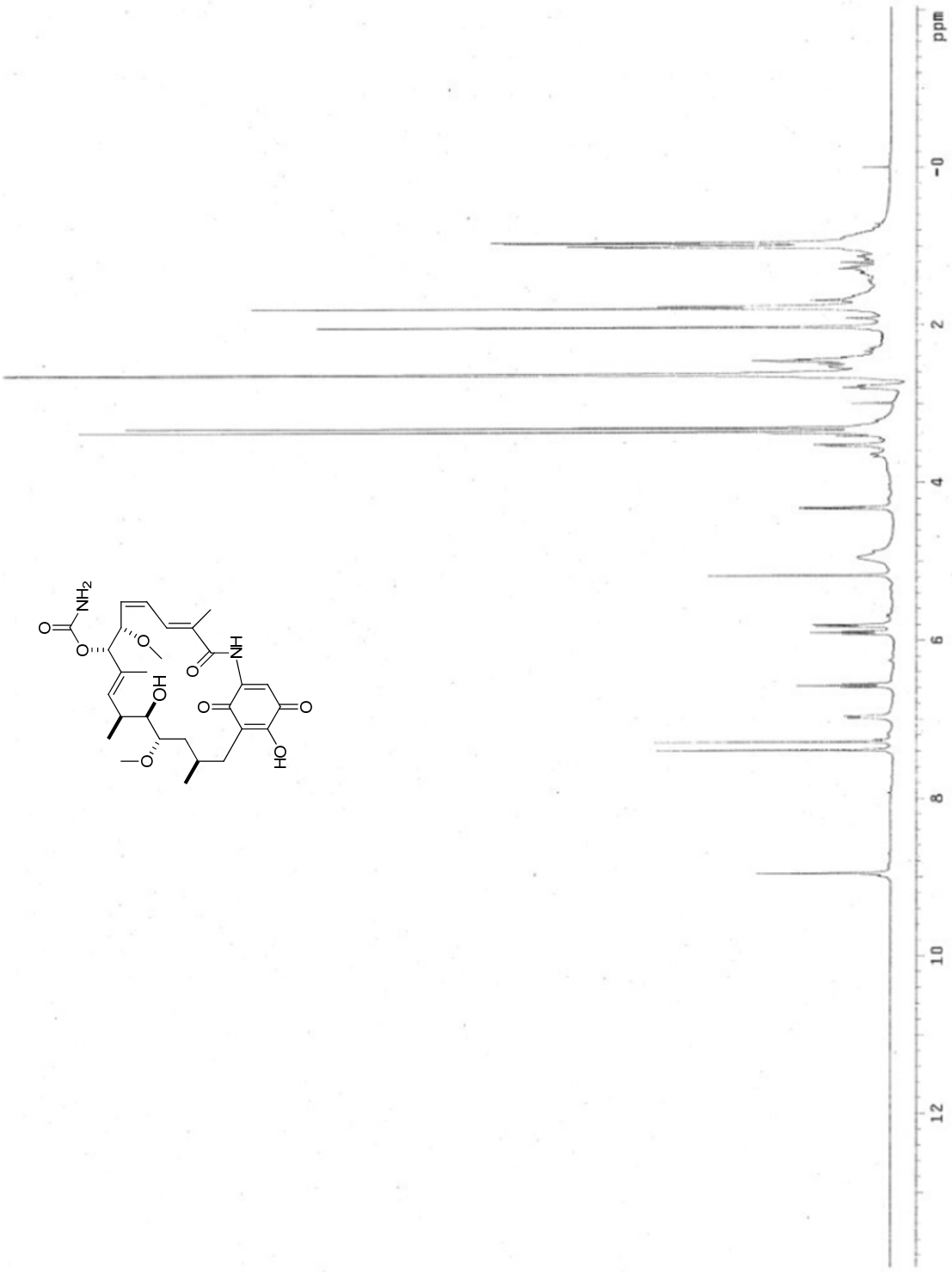


Figure S4. ^{1}H NMR spectrum of 17-O-demethyl-GDM (**4**) in $CDCl_3$

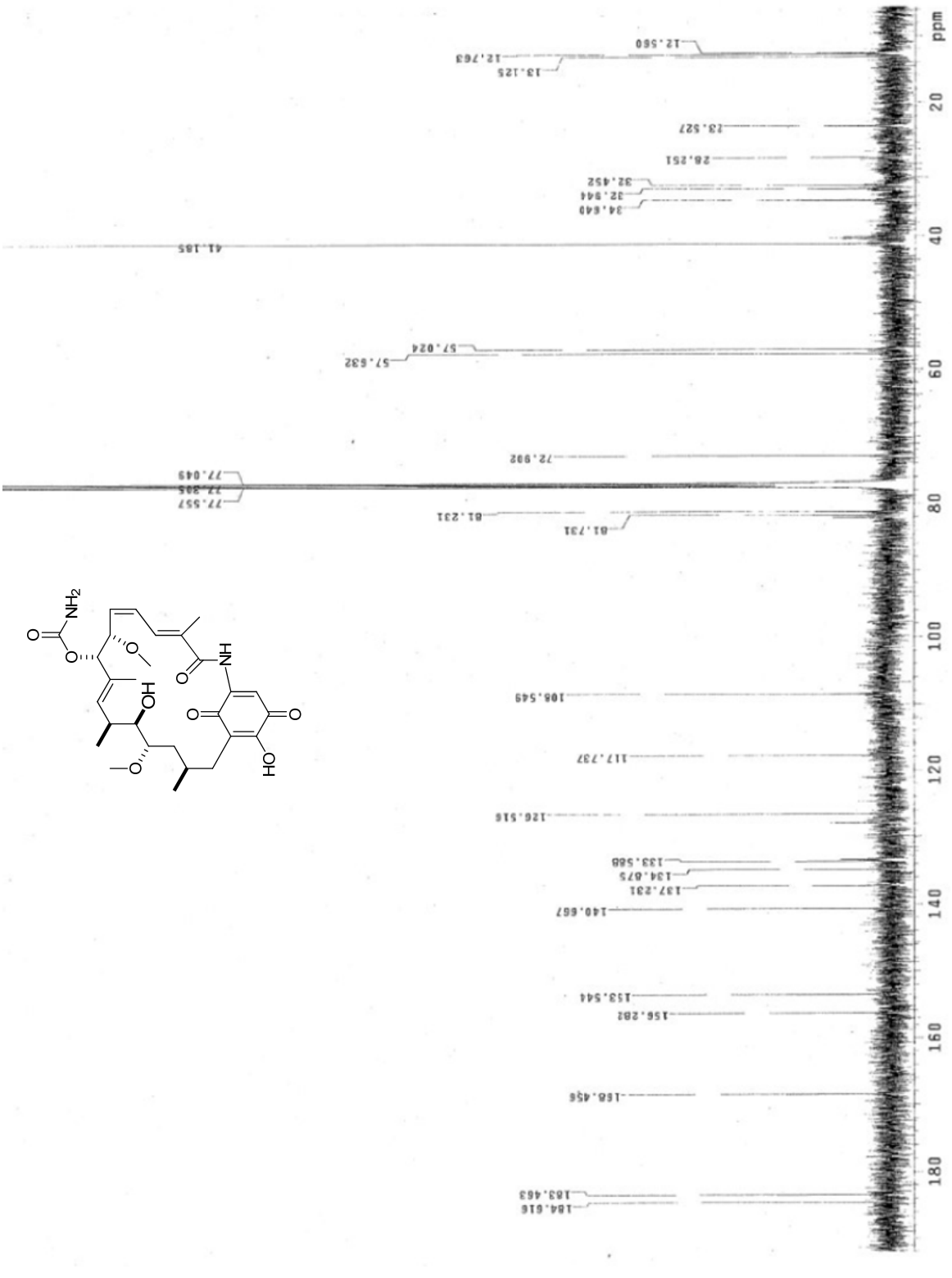


Figure S5. ^{13}C NMR spectrum of 17-O-demethyl-GDM (**4**) in $CDCl_3$

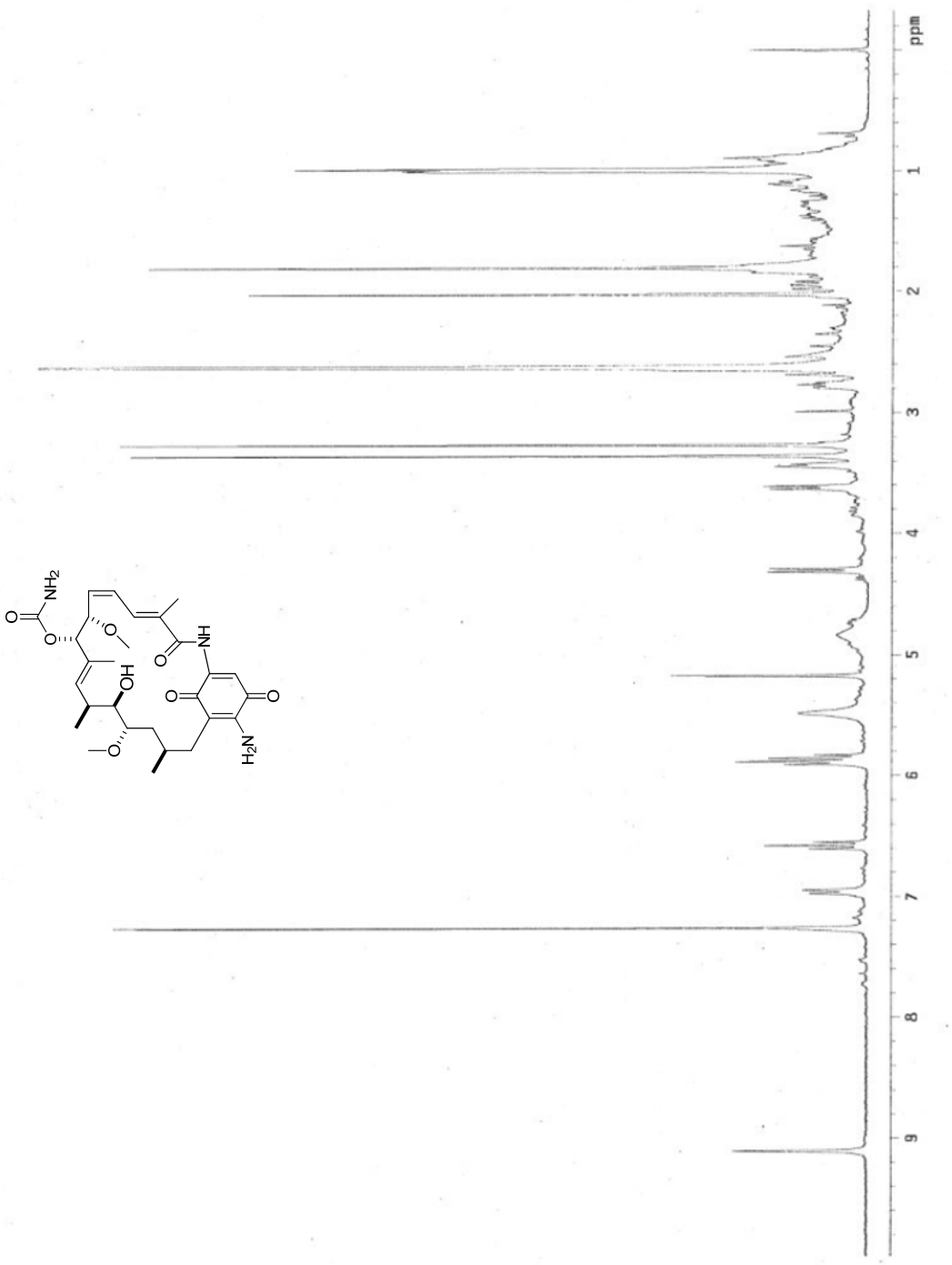


Figure S6. ^{1}H NMR spectrum of 17-amino-17-demethoxy-GDM (**2**) in $CDCl_3$

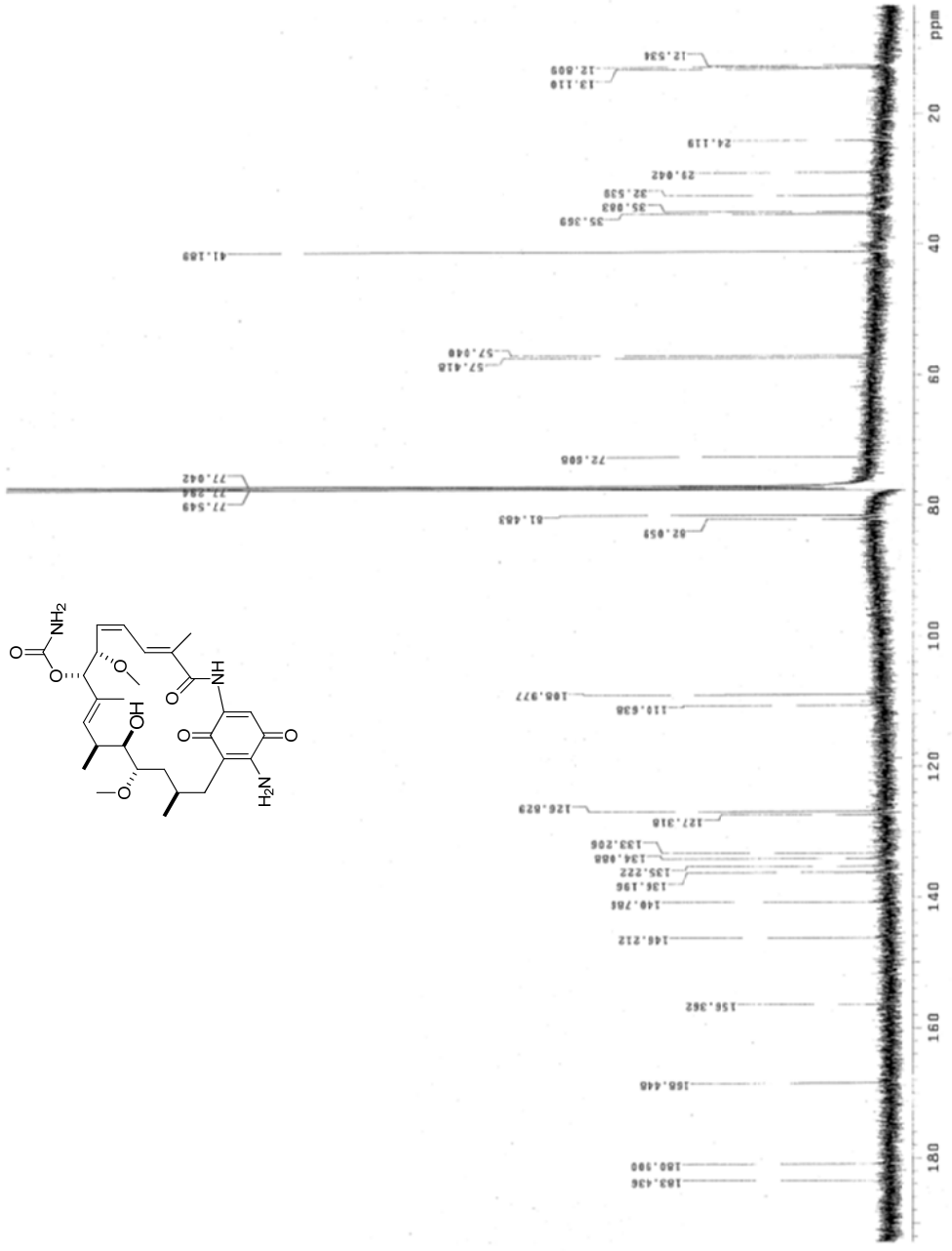


Figure S7. ^{13}C NMR spectrum of 17-amino-17-demethoxyl-GDM (**2**) in $CDCl_3$

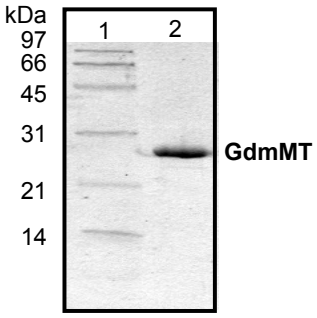


Figure S8. GdmMT purified from *E. coli* BL21/pBS17009 used in this study as judged upon 12% SDS-PAGE: lane 1, low molecular weight protein standards and lane 2, purified His6-tagged GdmMT with the predicted molecular mass of ~26 kDa.

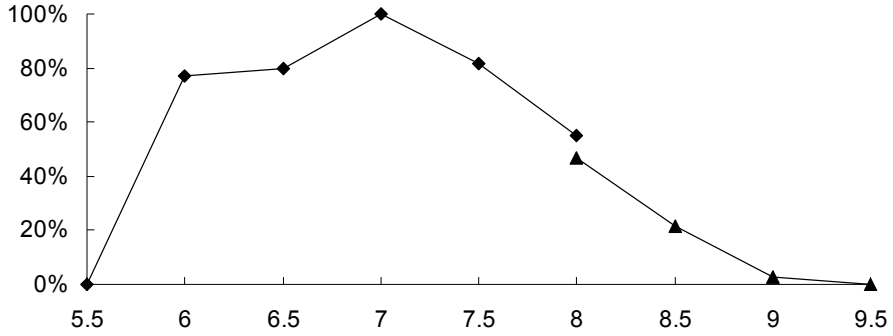


Figure S9. Effect of pH on the relative activity of GdmMT as the C-17 O-methyltransferase as determined with 17-O-demethyl-GDM (**4**) and SAM as substrates in 50 mM potassium phosphate (♦, pH 5.5-8.0) or 50 mM bis-tris-HCl (▲, pH 8.0-9.5).

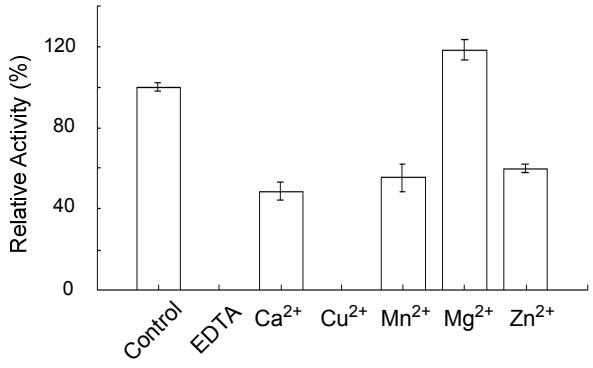


Figure S10. Effect of 5 mM divalent metal ions on the relative activity of GdmMT as the C-17 O-methyltransferase as determined with 17-O-demethyl-GDM (**4**) and SAM as substrates in 50 mM potassium phosphate, pH 7.0.